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Effects of linker-insertion mutations in herpes simplex virus 1 gD on glycoprotein-induced fusion with cells expressing HVEM or nectin-1

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Abstract

Several cell surface molecules, including HVEM and nectin-1, can serve as entry receptors for herpes simplex virus (HSV) and as receptors for virus-induced or viral glycoprotein-induced cell fusion. The viral ligand for these receptors is the HSV envelope glycoprotein gD. A set of linker-insertion and deletion mutants of HSV type 1 (HSV-1) gD was analyzed for effects of the mutations on binding of gD to HVEM and nectin-1, on viral glycoprotein-induced cell fusion with target cells expressing HVEM or nectin-1 and on complementation of infectivity of a gD-null HSV-1 viral mutant. Insertions after amino acid 151 or 225 or deletion of amino acids 234–244 disrupted (i) binding of the mutant forms of gD to both receptors and (ii) functional interactions (cell fusion and complementation) with both receptors, but were without effect on cell surface expression. Insertions in the N-terminal domain of gD (after amino acid 12, 34 or 43) disrupted binding to HVEM and functional activities with HVEM, as expected from a previously reported X-ray structure of a gD–HVEM complex, but were without effect in the case of nectin-1. These and other results indicate that the mutations disruptive of interactions with both receptors probably affect conformations of contact sites that are different for each receptor.

Keywords: Linker-insertion mutations; Herpes simplex virus 1 gD; Nectin-1

Introduction

Five of the several glycoproteins in the herpes simplex virus (HSV) envelope have been shown to participate in HSV entry. Glycoproteins gC and/or gB mediate initial attachment of the virus to the cell through interactions with cell surface heparan sulfate (reviewed in Spear, 1993). Once the virus is attached, gD can bind one of its multiple cell surface entry receptors. Although the mechanisms are not clear, gB, gD and the gH–gL heterodimer act together to induce fusion of the viral envelope with a cell membrane, but only upon interaction of gD with a gD receptor (reviewed in Spear et al., 2000). HSV also has the ability to induce cell–cell fusion, which can result in the formation of syncytia, or large multi-nucleated cells. This allows the virus access to several nuclei in which it can replicate without having to re-infect cells. The same glycoproteins that have been shown to be required for HSV entry are also necessary and sufficient for HSV-induced cell–cell fusion. Cells expressing gB, gD and gH-gL can fuse with cells expressing the appropriate gD receptor (Davis-Poynter et al., 1994; Muggeridge, 2000; Pertel et al., 2001). Thus, the viral and cellular determinants of HSV-induced cell–cell fusion appear to be similar to those for viral entry.

Three classes of gD entry receptors have been described to date: HVEM (herpesvirus entry mediator), the nectins and 3-*O*-sulfotransferase-modified heparan sulfate (reviewed in Spear et al., 2000). HVEM, also known as HveA, ATAR or TNFRSF14, is a member of the tumor necrosis factor receptor family and has cysteine-rich pseudorepeats in its ectodomain that are characteristic of the family (Montgomery et al., 1996). Its natural ligands include LIGHT and lymphotoxin-α (Mauri et al., 1998). Its HSV ligand, gD, binds directly to residues in the N-terminal two pseudor-

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epeats of HVEM (Carfi et al., 2001). HVEM is expressed on a variety of cell types and can mediate the entry of both HSV types 1 and 2 (HSV-1 and HSV-2); it is the principal entry receptor used by HSV to infect certain cell types, such as T lymphocytes, but appears not to be critical for entry into a number of other cell types (Montgomery et al., 1996).

The nectins are members of the immunoglobulin (Ig) superfamily and are related to the poliovirus receptor. They have three Ig-like folds, V-like at the N terminus and C-like in the membrane-proximal two domains. There are at least four nectins (nectin-1, nectin-2, nectin-3, nectin-4), some of which are widely expressed on many cell types and localize to cadherin-based adherens junctions (reviewed in Takai and Nakanishi, 2003). Only nectin-1 and nectin-2, also known as HveC and HveB, respectively, have been shown to serve as HSV entry receptors (Cocchi et al., 1998b; Geraghty et al., 1998; Lopez et al., 2000; Warner et al., 1998). Nectin-1 can mediate the entry of HSV-1 and HSV-2, as well as the animal alphaherpesviruses pseudorabies virus (PRV) and bovine herpesvirus type 1. Nectin-2 mediates the entry of HSV-2 and PRV, but has only very weak entry activity for HSV-1. The N-terminal V-like domains of these molecules have been shown to bind to gD (Cocchi et al., 1998a; Geraghty et al., 2001; Krummenacher et al., 1998; Lopez et al., 2000; Martinez and Spear, 2001). Several isoforms of the heparan sulfate-modifying enzyme, 3-*O*-sulfotransferase, can generate sites in heparan sulfate for gD binding, allowing for entry of HSV-1 preferentially (Shukla et al., 1999; Xia et al., 2002).

HSV-1 gD is a type 1 membrane glycoprotein and has 369 amino acids after signal peptidase cleavage. It has three sites for N-linked glycosylation and three disulfide bonds. Crystal structures of a soluble truncated form of HSV-1 gD (N-terminal 285 amino acids) were recently solved, with gD alone or in complex with a soluble form of HVEM (Carfi et al., 2001). It was determined that an Ig-like fold with an unusual disulfide bonding pattern comprises the core of the gD N-terminal domain. This core is flanked by an N-terminal extension which contains the HVEM binding region and forms a hairpin structure when bound to HVEM, but is disordered in the crystals of gD alone. C-terminal of the Ig-fold is an alpha helix that is sandwiched between the N-terminal hairpin and the Ig-like core and appears to support the HVEM contact site. Although the precise binding sites for the other gD receptors have not yet been defined, recent results show that at least a portion of the contact region for nectin-1 must be downstream of amino acid 32 and therefore downstream of the N-terminal hairpin region (Yoon et al., 2003).

Mutations in gD that can reduce or enhance physical and functional interactions with some of the HSV entry receptors have been defined. Consistent with the X-ray structure of gD-HVEM complexes, certain amino acid substitutions in the HVEM contact regions (amino acids 7–15 and 24–32) can prevent binding of HSV-1 gD to HVEM and also prevent viral entry and cell fusion when HVEM is the entry/fusion receptor (Connolly et al., 2003; Montgomery et al., 1996; Pertel et al., 2001; Whitbeck et al., 1997; Yoon et al., 2003). Subsets of these substitutions in HSV-1 gD actually enhance viral entry and cell fusion via nectin-2 and/or inhibit viral entry and cell fusion via 3-*O*-sulfated heparan sulfate (Connolly et al., 2003; Pertel et al., 2001; Warner et al., 1998; Yoon et al., 2003). Another subset also enhances entry/fusion activity with nectin-2 and somewhat reduces, but does not eliminate, activity with HVEM (Connolly et al., 2003; Lopez et al., 2000; Yoon et al., 2003). However, these N-terminal substitutions in either HSV-1 or HSV-2 gD have no effect on binding to nectin-1 or viral entry and cell fusion via nectin-1 (Connolly et al., 2003; Geraghty et al., 1998; Krummenacher et al., 1998; Yoon et al., 2003). Finally, deletions encompassing one or both of the HVEM contact regions (amino acids 7–15 and 24–32) significantly reduce functional interactions (as assessed by cell fusion) of either HSV-1 or HSV-2 gD with all the entry/fusion receptors except nectin-1 (Yoon et al., 2003). Thus, interactions of gD with the various entry/fusion receptors are differentially affected by the mutations just described and none of the mutations described has any demonstrable effect on viral entry by, or cell fusion induced by, nectin-1.

A panel of gD linker-insertion mutants was previously constructed to identify functional regions of gD important for viral entry (Chiang et al., 1994). This study was undertaken before the discovery of the HSV entry receptors and made use of Vero cells, which express multiple primate forms of the HSV entry receptors. Here we describe further analysis of 21 of these mutants to determine which retain the ability to bind soluble forms of HVEM or nectin-1, to induce the fusion of cells expressing full-length HVEM or nectin-1 and to mediate entry of virus into cells expressing the full-length forms of HVEM and nectin-1. We have identified a region of gD that is critical for binding and functional interaction of gD with both HVEM and nectin-1.

Results

Cell surface expression of gD mutants

All the gD mutants used in this study had previously been shown to retain epitopes for at least two of seven monoclonal antibodies (mAbs) and to be expressed on the cell surface (Chiang et al., 1994), although cell surface expression was not quantitatively assessed. To quantitate cell surface expression under the conditions of the cell fusion assay used here, CHO-K1 cells were co-transfected with plasmids expressing HSV-1 gB, gH, gL and T7 polymerase, including also a plasmid expressing wild-type HSV-1 gD or one of the HSV-1 gD mutants listed in Table 1. After seeding into 96-well plates, the transfected cells were washed and incubated with an anti-gD rabbit serum and then fixed and incubated sequentially with a biotin-conjugated secondary antibody, streptavidin–horseradish

Table 1
gD linker-insertion and deletion mutants[a]

Plasmid	Mutation	Amino acids inserted	Plasmid	Mutation	Amino acids inserted
pRE4	None	none	D1-HC235	ins 225	GRSS
D1-H28[b]	ins 12[c]	GKIFP	pWW17[d]	del 234–244	–
D1-N29	ins 34	GKIFL	D1-H24	ins 243	GRSS
D1-H27	ins 43	EDLP	D1-H54	ins 246a	GKIFP
D1-H78	ins 77a	GKIFP	D1-N1	ins 246b	EDLP
D1-N65	ins 83	GRSS	D1-H26	ins 277	GKIFP
D1-H104	ins 84	GKIFP	D1-N2	ins 287	EDLP
D1-N22	ins 125	GRSS	D1-H15	ins 310	GKIFRKIFP
D1-H98	ins 126	GKIFP	pHC238	del 277–290	GKIFP
D1-N37	ins 151	WKIFL	pHC240	del 290–300	GKIFP
D1-N119	ins 187	GRSS	pHC241	del 290–310	GKIFP

[a] Described by Chiang et al. (1994).
[b] D1 refers to the gD gene of HSV-1. The letter after the dash refers to the restriction enzyme used (H, *Hae*III; N, *Nla*IV).
[c] ins, insertion-number indicates the amino acid residue preceding the insertion; del, deletion of the residues indicated.
[d] Constructed as described by Cohen et al. (1988).

peroxidase and substrate. The results presented in Fig. 1 show that all the gD mutants were expressed on the surfaces of CHO cells at or near wild-type levels, with the exception of ins246b and ins277.

Binding of soluble receptors to gD mutants

We used soluble secreted forms of entry/fusion receptors, hybrids of the HVEM and nectin-1 ectodomains fused to the Fc region of rabbit IgG (HVEM:Fc and nectin-1:Fc), to test whether the mutant forms of gD retained receptor-binding activity. For these studies, CHO-K1 cells were co-transfected with plasmids expressing gB, gH, gL and T7 polymerase, including also a plasmid expressing one of the gD mutants or wild-type gD. After seeding in 96-well plates, the transfected cells were washed and incubated with HVEM:Fc or nectin-1:Fc at 10 ng/ml, and then fixed before incubation with a detection system specific for the Fc domain. The results of these binding studies are shown in Figs. 2 and 3. The gD mutants fell into three categories, according to their ability to bind HVEM:Fc and nectin-1:Fc. Mutants in the first category (ins77, ins83, ins84, ins187, ins277, ins287, ins310, del277–290, del290–300, del290–310) retained the ability to bind both receptors at levels greater than 50% than that observed for wild-type gD. Mutants in the second category (ins12, ins34, ins43, ins125, ins126, ins243, ins246a) were impaired for binding to HVEM:Fc, but not to nectin-1:Fc. Mutants in the third category (ins151, ins225, del234–244, ins246b) were

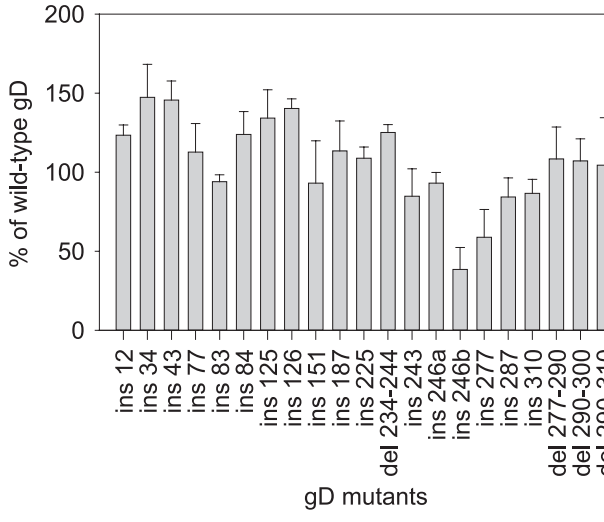

Fig. 1. Cell surface expression of HSV-1 gD mutants. CHO-K1 cells were co-transfected with plasmids expressing HSV-1 gB, gH, gL and T7 polymerase, including also a plasmid expressing one of the HSV-1 gD mutants, wild-type gD or an empty control vector. After seeding into 96-well plates, the transfected cells were incubated with an anti-gD rabbit serum, then washed and fixed and incubated with an HRP-based detection system. Percent of wild-type gD = {(HRP units for mutant gD − HRP units for control)/(HRP units for wild-type gD − HRP units for control)} × 100. The results presented here are the means of three independent experiments (each done in triplicate) with standard deviation.

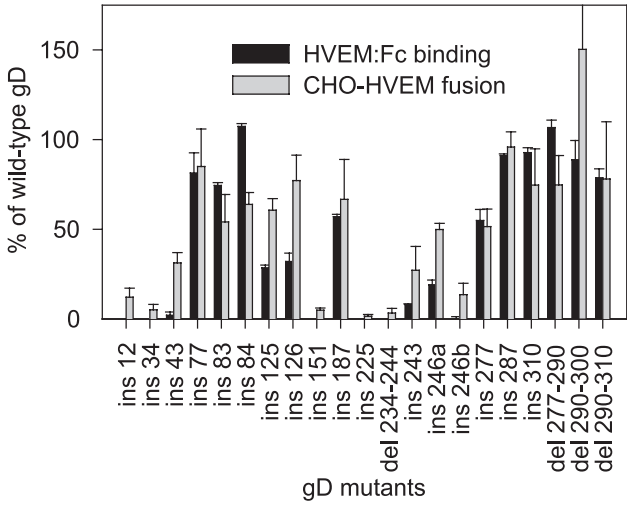

Fig. 2. Binding and fusion activities of gD mutants with HVEM. Each mutant was tested for binding (black bars) and fusion (gray bars) activity in comparison with wild-type gD. CHO-K1 cells were co-transfected with plasmids expressing HSV-1 gB, gH, gL and T7 polymerase, including also a plasmid expressing one of the HSV-1 gD mutants, wild-type gD or an empty control vector. To determine binding activity, the transfected cells were seeded in 96-well plates, incubated with HVEM:Fc (10 ng/ml), washed, fixed and incubated with an HRP-based detection system. Percent of wild-type gD = {(HRP units for mutant gD − HRP units for control)/(HRP units for wild-type gD − HRP units for control)} × 100. HRP unit values (OD_{370}) for wild-type gD ranged from 0.332 to 0.353 and for the negative control from 0.125 to 0.138. To measure fusion activity, cells expressing wild-type or a mutant form of gD, along with gB, gH-gL and T7 polymerase, were mixed with CHO-HVEM cells transfected with a T7 luciferase plasmid. Luciferase activity was quantitated as a measure of fusion. Percent of wild-type gD = {(luciferase activity for mutant gD − luciferase activity for control)/(luciferase activity for wild-type gD − luciferase activity for control)} × 100. Values (luciferase activity in arbitrary units) for wild-type gD ranged from 4743 to 10,097 and for the negative control from 57 to 142. Means and standard deviations for triplicate determinations in a single experiment are shown. Similar results were obtained in two other experiments.

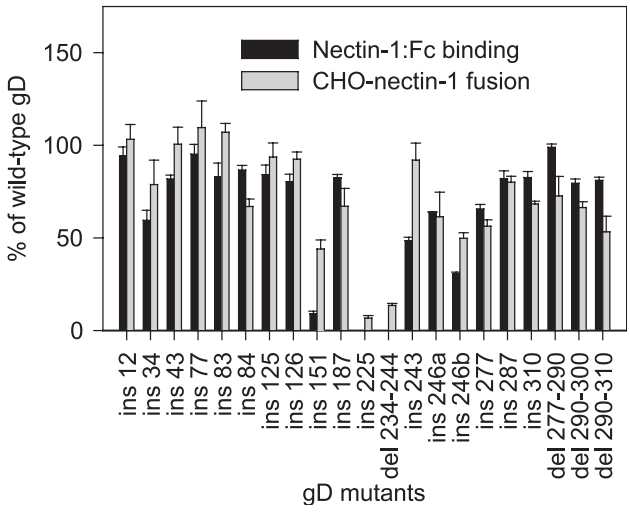

Fig. 3. Binding and fusion activities of gD mutants with nectin-1. Each mutant was tested for binding (black bars) and fusion (gray bars) activity in comparison with wild-type gD. Methods and data analysis are as described in the legend to Fig. 2 except that nectin-1:Fc was used for the binding assay and CHO-nectin-1 cells as the targets in the cell fusion assay. For the binding assay, HRP unit values (OD_{370}) for wild-type gD ranged from 1.007 to 1.105 and for the negative control from 0.293 to 0.332. For the cell fusion assay, values (luciferase activity in arbitrary units) for wild-type gD ranged from 56,659 to 60,395 and for the negative control from 666 to 2093. Means and standard deviations for triplicate determinations in a single experiment are shown. Similar results were obtained in two other experiments.

impaired for binding to both receptors. The results obtained with mutant ins246b might be explained in part by its reduced or altered presentation on cell surfaces (Fig. 1). None of the mutants was impaired for binding to nectin-1 while also retaining significant binding activity for HVEM.

Functional analysis of the gD mutants

The effects of the mutations on gD function were assessed first in a virus-free, quantitative cell fusion assay. This assay depends on the expression of four HSV-1 glycoproteins (gB, gD, gH and gL) and T7 polymerase in one cell population (effector cells) and on expression of an entry/fusion receptor, and presence of a luciferase gene driven by the T7 promoter, in a second cell population (target cells). The effector cells were co-transfected with plasmids expressing HSV-1 gB, gH, gL and T7 polymerase, including also a plasmid expressing one of the gD mutants or wild-type gD, and then mixed with target cells expressing HVEM or nectin-1. The extent of fusion was assessed by quantitating luciferase expression. The results obtained are presented in Figs. 2 and 3 along with the receptor-binding results just described. In general, cell fusion activity correlated with receptor-binding activity. In some cases, greater cell fusion activity (normalized to control values obtained with wild-type gD) was retained than might have been predicted on the basis of the receptor-binding results. This may be explained in part by potential differences between soluble and membrane-bound forms of receptor in ability to interact with mutant forms of gD. Fig. 2 shows that mutations in the N-terminal region of gD (ins12, ins34 and ins43) severely reduced the fusion of effector cells with the HVEM-expressing target cells (<50% the level observed with wild-type gD), as did mutations in other domains of gD (ins151, ins225, del234–244, ins243 and ins246b). These same mutations severely disrupted binding with HVEM:Fc.

On the other hand, only three mutations (ins151, ins225 and del234–244) disrupted both the binding of nectin-1:Fc and the fusion of effector cells with nectin-1-expressing target cells. Mutation ins151 had a less drastic effect on fusion with nectin-1-expressing target cells than with HVEM-expressing target cells, whereas mutations ins225 and del234–244 drastically reduced fusion with both target cells. In a recent study, many of the same gD mutants used here were expressed in baby hamster kidney (BHK) cells, along with HSV-1 gB, gH and gL, to assess cell fusion activity. Binding of a soluble nectin-1:Fc hybrid to BHK cells expressing the various gD mutants was also quantitated. The results obtained were different from those presented in Fig. 3, most notably in findings of reduced cell fusion and nectin-1-binding activities for the N-terminal insertion mutants, ins12, ins34, ins43 (Zhou et al., 2003). Because the levels of gD cell surface expression were not assessed in the cited study, the differences noted could be trivial and relate only to efficiencies of transfection and expression from the various plasmids. The differences in cell fusion results could also be explained by expression of human nectin-1 in the target cells used here (Fig. 3) and BHK entry/fusion receptors, which have not yet been fully identified or characterized, in the target cells used in the cited study. The differences in nectin-1:Fc binding results could be due to various factors, including use of different hybrid forms of nectin-1 at different concentrations (nectin-1:Fc-rabbit at 10 ng/ml in this study versus nectin-1:Fc-human at 600 ng/ml in the cited study) or expression of the gD mutants in different cell types. BHK cells express functional HSV entry receptors, whereas CHO cells have only very weak endogenous receptors for HSV-1. Through an interference phenomenon (Geraghty et al., 2000), the endogenous receptors in BHK cells could compete with soluble nectin-1 for binding to some of the gD mutants.

To address whether the mutant forms of gD were functional for viral entry via HVEM or nectin-1, we used the complementation assay previously described (Chiang et al., 1994) but with several modifications. Vero cells were transfected with a plasmid expressing wild-type gD or one of the mutant forms of gD and then treated with cycloheximide (50 μg/ml) to delay the synthesis of gD until after the cells had been infected with a complemented gD-null virus, to prevent gD-mediated interference (Geraghty et al., 2000). At 24 h after transfection, the cells were washed free of cycloheximide and infected with the gD-null virus, HSV-1(KOS)gD6. Complemented virus particles were then har-

vested and used to infect CHO-HVEM and CHO-nectin-1 cells. The results are presented in Figs. 4 and 5, in comparison with the cell fusion results from Figs. 2 and 3. In general, the complementation results paralleled the cell fusion results. Mutations that impaired cell fusion activity the most also significantly reduced complementation of viral infectivity, in a receptor-dependent fashion. With some exceptions, the complementation results obtained here also paralleled the complementation results obtained previously with Vero cells (Table 2). Most of the exceptions were results obtained with the N-terminal mutations (ins12, ins34, ins43). On the basis of their complementation activities, the gD mutants fall into four categories: (1) similar complementation activities with all three cell types; (2) similar only with CHO-HVEM cells and Vero cells; (3) similar only with CHO-nectin-1 cells and Vero cells; and (4) similar only with CHO-HVEM and CHO-nectin-1 cells. Vero cells express the monkey forms of both HVEM and nectin-1 (Foster et al., 1999; Milne et al., 2001), and possibly other entry receptors, but features of HSV-1 gD that govern interactions with monkey entry receptors are not known.

Retention of cell fusion activity by some of the gD mutants analyzed here did not necessarily predict comparable complementation of viral infectivity (Figs. 4 and 5).

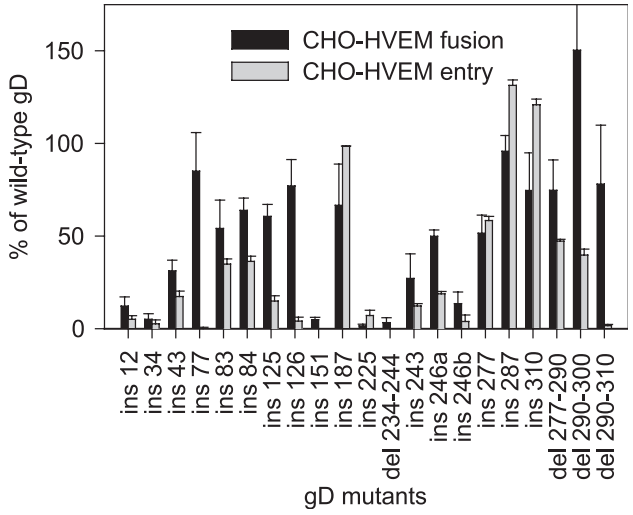

Fig. 4. Ability of gD mutants to mediate viral entry into CHO-HVEM cells. Each gD mutant was tested for ability to complement the entry defect of a gD-null HSV-1 viral mutant (gray bars) in comparison with wild-type gD. Results are presented along with the cell fusion results from Fig. 2 (black bars), for comparison. Vero cells were transfected with a plasmid expressing wild-type gD or one of the mutant forms of gD and treated with cycloheximide (50 μg/ml). After 24 h, the cells were washed and infected with 20 PFU/cell of HSV-1(KOS)gD6. After an additional 24 h, complemented virus particles were harvested and used to inoculate CHO-HVEM cells. After 6 h, the cells were lysed for the quantitation of β-galactosidase activity (OD_{410} of ONPG reaction product) as a measure of viral entry. Percent of wild-type gD = {(ONPG units for mutant gD − ONPG units for control)/(ONPG units for wild-type gD − ONPG units for control)} × 100. Values for wild-type gD ranged from 0.145 to 0.150 and for the negative control from 0.099 to 0.106. Results presented are the means of triplicate determinations.

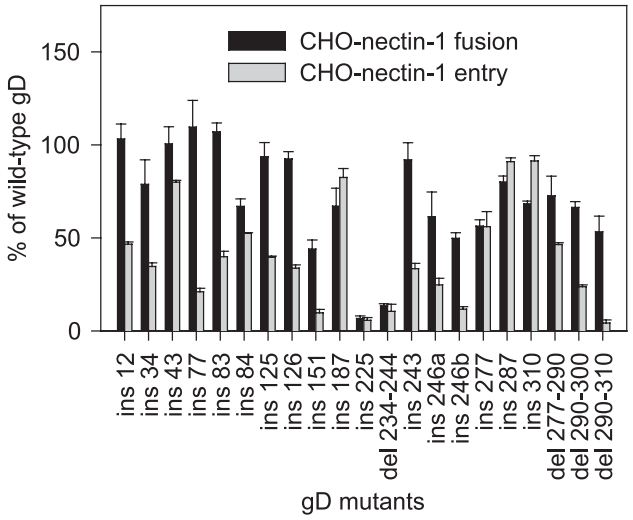

Fig. 5. Ability of gD mutants to mediate viral entry into CHO-nectin-1 cells. Each gD mutant was tested for ability to complement the entry defect of a gD-null HSV-1 viral mutant (gray bars) in comparison with wild-type gD. Results are presented along with the cell fusion results from Fig. 3 (black bars), for comparison. Methods and data analysis are as described in the legend to Fig. 4 except that the complemented viruses were tested for entry into CHO-nectin-1 cells. Values (OD_{410} of ONPG reaction product) for wild-type gD ranged from 0.151 to 0.153 and for the negative control from 0.101 to 0.103. Results presented are the means of triplicate determinations.

Most of the insertions upstream of residue 151 impaired complementation of viral infectivity more than cell fusion activity. Also, del277–290, del290–300 and, in particular, del290–310 reduced complementation but not cell fusion

Table 2
Complementation assays of linker-insertion mutants

Mutation	Complementation (% of control with wild-type gD)		
	CHO-HVEM cells	CHO-nectin-1 cells	Vero cells[a]
ins 12	5	47	96
ins 34	3	35	2
ins 43	17	80	9
ins 77a	0	21	16
ins 83	35	40	77
ins 84	36	53	115
ins 125	15	40	21
ins 126	4	34	17
ins 151	0	10	0
ins 187	98	83	79
ins 225	7	6	0
del 234–244	0	11	nd
ins 243	12	33	3
ins 246a	19	25	1
ins 246b	4	12	1
ins 277	58	56	44
ins 287	131	91	59
ins 310	121	91	105
del 277–290	47	46	12
del 290–300	40	24	7
del 290–310	2	4	14

[a] Data from Chiang et al. (1994).

activity. Similar results were obtained previously for these deletion mutants when tested on Vero cells (Table 2). Reduced complementation could have resulted from impaired incorporation of the mutant forms of gD into virions or reduced entry activity of the incorporated gD or both. The experiments done to date cannot differentiate among these possibilities. This will require transfer of interesting mutations to the viral genome so that purified progeny produced in the absence of complementation can be analyzed as to composition and specific infectivity.

Discussion

Results presented here identify regions of HSV-1 gD that are critical for functional interactions with the two major HSV-1 entry receptors, HVEM and nectin-1, and also provide additional evidence that the N-terminal domain of gD is critical for interactions with HVEM, but not with nectin-1. The mutations ins151, ins225 and del234–244 disrupted binding of soluble HVEM:Fc and nectin-1:Fc to cells expressing the gD mutants and also disrupted cell fusion activity and complementation activity of the mutants whether the fusion/entry receptors were HVEM or nectin-1. As previously shown (Chiang et al., 1994; Muggeridge et al., 1990), ins151, ins225 and del234–244 also disrupted complementation activity when the fusion/entry receptors were those expressed in Vero cells. These results are not due to absence of gD cell surface expression or loss of epitopes recognized by a polyclonal antiserum, as shown in Fig. 1. Also, ins225 and del234–244 retained epitopes for 5/7 and 6/7 mAbs tested, respectively (Chiang et al., 1994; Muggeridge et al., 1990). Ins151, on the other hand, retained epitopes for only 2/7 mAbs, indicating significant changes in conformation (Chiang et al., 1994).

Consideration of our results and the X-ray structure of gD (Carfi et al., 2001) indicates that the mutations most disruptive of binding to both HVEM and nectin-1, and of functional activity with HVEM and nectin-1, are predicted to disrupt critical secondary structural elements of gD (Fig. 6). Ins151 would bisect the E beta strand of the Ig fold. Ins225 would interrupt the N-terminal end of alpha helix 3, which is sandwiched between the N-terminal domain and the Ig fold. Del234–244 removes the C-terminal half of alpha helix 3. It seems likely that these mutations alter the conformation of gD so as to preclude binding of both HVEM and nectin-1, without affecting the folding and processing necessary for transport to the cell surface. Only two other mutations studied here are predicted to disrupt similar structural elements. Ins77 and ins83 should affect alpha helix 1, a small helix within a large surface loop. These latter insertions had little effect on binding or cell fusion with nectin-1 as receptor, and caused only marginal impairment of these activities with HVEM. Ins77 did, however, impair complementation of viral entry via both

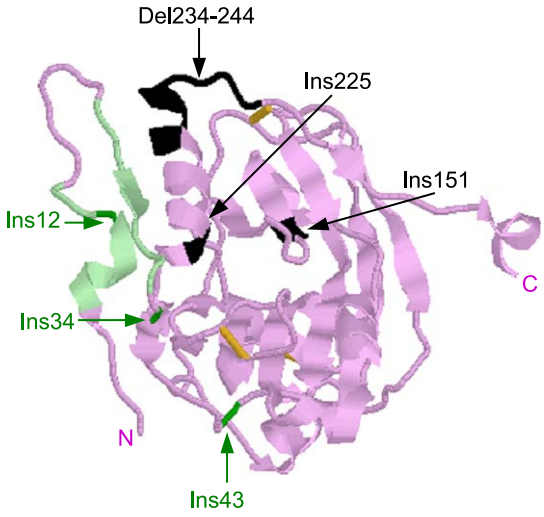

Fig. 6. Structure of HSV-1 gD and location of insertions/deletions that disrupted functional interactions with HVEM alone (dark green) or with both HVEM and nectin-1 (black). This ribbon backbone trace is based on coordinates deposited in the Protein Data Bank (1JMA) for the structure of gD in complex with HVEM (Carfi et al., 2001). HVEM is not shown here. The HVEM contact regions (7–15 and 24–32) are colored light green. N = N terminus; C = C terminus.

receptors. All the other insertions or deletions analyzed here were in regions of loops or were downstream of the portion of gD that was ordered in the X-ray structure (downstream of amino acid 259). Because the mutants studied here passed the tests of epitope retention and transport to the cell surface, it is perhaps not surprising that few of the insertions or deletions were in structural elements such as beta strands and alpha helices.

Our results are consistent with previous studies that mapped epitopes of gD critical for interactions with HVEM and nectin-1. mAbs specific for several distinct or overlapping antigenic sites were tested for ability to block the binding of HVEM or nectin-1 to HSV-1 virions. Only mAbs 1D3 (site VII) and DL11 (site Ib) inhibited the binding of HVEM to virions (Nicola et al., 1998). The linear epitope recognized by 1D3 has been mapped to amino acids 11–19 and the conformational epitope recognized by DL11 to regions upstream of amino acid 258 (Cohen et al., 1992). Only mAbs HD1 (site Ia) and DL11 (site Ib) inhibited the binding of nectin-1 to virions (Krummenacher et al., 1998). Sites Ia and Ib are overlapping conformational epitopes with site Ia mapping to a region upstream of amino acid 226 (Cohen et al., 1992). It is of interest that mutation ins12 reduced the binding of mAb 1D3 only partially, whereas all the other mutations tested here, including those that inhibited interaction with HVEM, had no effect on 1D3 binding (Chiang et al., 1994). Mutations ins151, ins225 and del234–244, all of which inhibited functional interactions with both HVEM and nectin-1, abrogated the binding of mAb DL11, but only ins151 prevented binding of mAb HD1 (Chiang et al., 1994; Muggeridge et al., 1990). Ins151 also inhibited the binding of three other mAbs specific for

other conformational antigenic sites (Chiang et al., 1994), indicating that this mutation altered the overall conformation of gD despite its lack of effect on cell surface expression and only partial inhibition of cell fusion activity with nectin-1.

The results presented here have not only identified regions of gD that are critical for integrity of the contact regions for both HVEM and nectin-1, but also provide additional evidence that the primary contact regions are largely non-overlapping. Insertions in the N terminus of gD (ins12, ins34, ins43) disrupted physical and functional interactions with HVEM, as predicted from the X-ray structure of the HVEM–gD complex, but were without effect on binding of gD to nectin-1, cell fusion with nectin-1 as receptor and complementation of viral entry via nectin-1. This is consistent with previous findings that amino acid substitutions in the N-terminal domain of gD, and deletions encompassing amino acids 7–32 of gD, disrupt interactions with HVEM, but not nectin-1 (Connolly et al., 2003; Geraghty et al., 1998; Krummenacher et al., 1998; Montgomery et al., 1996; Whitbeck et al., 1997; Yoon et al., 2003). Thus, while the principal gD–HVEM interface is between amino acids 7 and 32, the principal gD–nectin-1 interface must be downstream of amino acid 32. The possibility exists that there may be secondary overlapping interfaces with HVEM and nectin-1, one interpretation of the findings described above that mAb DL11 can block the binding of both HVEM and nectin-1 to virions. Other interpretations of the effects of DL11 are that (i) binding of DL11 to its epitope sterically hinders interaction with both receptors even though their contact interfaces are different or (ii) binding of DL11 causes conformational changes that alter distinct and different interfaces for each of the receptors. If there are no overlaps in the contact interfaces for HVEM and nectin-1, then the mutations described above that disrupt interactions with both HVEM and nectin-1 must alter conformation of both of the non-overlapping primary interfaces, including the N-terminal hairpin structure important for binding to HVEM.

It is of interest that none of the mutations analyzed here disrupted cell fusion activity or complementation of viral entry without also disrupting binding to receptor. If the binding of gD to receptor leads to interaction of gD with one of the other HSV glycoproteins known to be required for membrane fusion (gB, gH or gL), it might be expected that there is an interface on gD for one of the other viral glycoproteins. Disruption of this interface would be predicted to inhibit cell fusion without necessarily affecting binding of gD to receptor. This possibility is not ruled out by our results because the gD open reading frame was not saturated with mutations that were suitable for analysis and because deletions or insertions might cause changes too drastic to permit identification of a domain that is critical only to events occurring after binding of gD to receptor. Point mutations, generated with guidance from the X-ray structure, may permit identification of regions in gD that are critical for cell fusion activity but not for receptor binding.

Materials and methods

Cells and viruses

CHO-K1 cells were provided by J. Esko (University of California at San Diego). CHO-K1 cells stably expressing HVEM, designated CHO-HVEM35 cells, were obtained by transfection with plasmid pBEC28 (see below) and selection in medium containing G418 at 500 μg/ml (R.I. Montgomery and P.-Y. Lim, manuscript in preparation). Nectin-1-expressing CHO cells were previously described (Geraghty et al., 1998; Montgomery et al., 1996). All CHO cell lines were passaged in Ham's F12 medium (Gibco BRL) supplemented with 10% fetal bovine serum (FBS) and antibiotics, including G418 (400 μg/ml) for stably transfected cells. HSV-1(KOS)gD6 is a gD-negative virus in which the gD open reading frame has been replaced by the *lacZ* gene under control of the CMV IE promoter (Warner et al., 1998). Stocks of this gD-negative mutant were propagated on the complementing VD60 cells (Ligas and Johnson, 1988).

Plasmids

Plasmids expressing HSV-1(Patton) gD linker-insertion and deletion mutants, which were generously provided by G. Cohen and R. Eisenberg (University of Pennsylvania, Philadelphia), were previously described (Chiang et al., 1994; Cohen et al., 1988) and are listed in Table 1. For construction of plasmid pBEC28, the open reading frame for HVEM was amplified by polymerase chain reaction (PCR) from pBEC10 (Montgomery et al., 1996) using a 3′ primer in which the stop codon was replaced with GAATTCGC. The resulting PCR product was digested with *Nhe*I and *Eco*RI and then cloned into the same sites present in the pcDNA3.1mycHIS B vector (Invitrogen, Inc.). Plasmid pBL58, expressing the human HVEM ectodomain fused to the hinge, CH_2 and CH_3 domain of the rabbit IgG heavy chain (HVEM:Fc) was previously described (Montgomery et al., 1996). Plasmid pBL60 has the HVEM:Fc hybrid open reading frame from pBL58 cloned into a derivative of pPUR (Clontech), in which the cytomegalovirus promoter for the puromycin-resistance gene was replaced with the Rous sarcoma virus promoter. Plasmid pBG37 expresses the equivalent human nectin-1:rabbit IgG fusion protein (nectin-1:Fc). It was constructed by PCR amplification of the nectin-1 ectodomain (nucleotides encoding the first 336 amino acids including the cleavable signal sequence) and ligation to the Fc region of rabbit IgG by a *Bam*HI site such that amino acid F336 of nectin-1 is fused to a linker encoding two amino acids (GS) and then to amino acid A175 of rabbit IgG (GenBank accession number

K00752.1). The hybrid open reading frame was inserted into pcDNA3 (N.A. Jones and R.J. Geraghty, manuscript in preparation).

Preparation of HVEM:Fc and nectin-1:Fc

A stable cell line that secretes HVEM:Fc was produced by transfection of CHO-K1 cells with pBL60 and selection in medium containing puromycin (5 μg/ml). The cells were plated in complete medium (Ham's F12 plus 10% FBS) and, 24 h later, were washed and transferred to Opti-MEM (Gibco BRL). Culture supernatants containing the secreted HVEM:Fc were collected 48 h later. To produce the nectin-1:Fc protein, PEAK cells (Edge Biosystems) were transfected with the plasmid pBG37 by the calcium phosphate method in DMEM containing 5% FBS. Twenty-four hours after transfection, the cells were washed and incubated with Opti-MEM and culture supernatants were collected 48 h later. The culture supernatants containing the secreted proteins were clarified by low-speed centrifugation and the concentration of the receptor:Fc hybrid proteins was determined by enzyme-linked immunosorbent assay (ELISA) with reagents that detected the rabbit Fc region.

Cell enzyme-linked immunosorbent assay (CELISA)

Subconfluent CHO-K1 cells were transfected with plasmids expressing the HSV-1 fusion-inducing glycoproteins and T7 RNA polymerase using Lipofectamine reagent in Opti-MEM. Individual wells of six-well plates were transfected with 0.5 μg of each plasmid, keeping the total amount of DNA at 2.5 μg per well by the addition of empty vector DNA, if necessary. After incubation at 37°C in 5% CO_2 for 8 h, the transfection mixture was removed and Ham's F12 medium containing 10% FBS was added. The cells were incubated for an additional 4 h before detaching and replating into 96-well plates. After incubation for 18 h, the cells were washed with phosphate-buffered saline (PBS) and then incubated with primary antibody, HVEM:Fc or nectin-1:Fc diluted in PBS containing 3% bovine serum albumin. Rabbit polyclonal anti-gD antibody, R7, was diluted 1:10,000; HVEM:Fc and nectin-1:Fc supernatants were used at 10 ng of receptor:Fc hybrid per milliliter. Subsequently, the cells were washed, fixed with PBS containing 2% formaldehyde and 0.2% glutaraldehyde, and incubated sequentially with biotinylated anti-rabbit IgG (Sigma), Amdex streptavidin-conjugated horseradish peroxidase (HRP; Amersham) and HRP substrate (BioFx Lab). Binding was monitored at 370 nm in a Victor Wallac spectrophotometer (Perkin-Elmer).

Cell fusion assay

The assay conditions and plasmids used were as previously described (Pertel et al., 2001), except that the gD-expressing plasmid used previously was replaced with one of the gD-expressing plasmids listed in Table 1. CHO-K1 effector cells were transfected with the plasmids expressing the HSV-1 fusion glycoproteins (gB, gD, gH and gL) and T7 RNA polymerase. Target CHO-HVEM or CHO-nectin-1 cells were transfected with the plasmid expressing luciferase under control of the T7 promoter. After incubation at 37°C in 5% CO_2 for 8 h, the transfection mixture was removed and Ham's F12 medium containing 10% FBS was added. The cells were incubated for an additional 4 h before detaching and replating into 96-well plates. Effector and target cells were mixed in a 1:1 ratio, and co-cultivated for 18 h. Luciferase activity was quantitated using the Luciferase Reporter Assay System (Promega).

Complementation assay

The procedure was performed as previously described (Chiang et al., 1994) with several modifications. Vero cells were transfected using Lipofectamine in Opti-MEM (Gibco BRL) with gD expression plasmids or an empty vector. After 6 h, the transfection mixture was removed and DMEM containing 10% FBS and cycloheximide (50 μg/ml) was added. Then, 24 h after transfection, the cells were washed with DMEM containing 10% FBS and incubated with 20 PFU/cell of complemented HSV-1(KOS)gD6 virus for 2 h at 37°C. The medium was removed and the extracellular virus was inactivated by the addition of citrate buffer (pH 3.0) for 1 min followed by two washes with PBS supplemented with 1% glucose and 1% calf serum. The cells were overlaid with fresh DMEM containing 1% serum and incubated for 24 h at 37°C. The infected cells were harvested by scraping into the culture supernatant. The cells were disrupted by sonication, and debris removed by low-speed centrifugation. Samples of the complemented viruses were added in triplicate to CHO-HVEM and CHO-nectin-1 cells grown in 96-well plates and incubated for 6 h. Cells were washed, permeabilized and incubated with β-galactosidase substrate, *O*-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma) as described (Montgomery et al., 1996). The reaction was monitored at 405 nm to quantitate viral entry.

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